MHC class II antigen presentation by B cells in health and disease
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Chapter 1

General introduction

The immune system protects us from harmful microbial infections. Such protection results from the interplay between innate and adaptive (acquired) immunity, both of which involve differential recognition of self from infectious non-self. While innate immunity relies upon antigen-nonspecific pattern-recognition receptors to broadly sense offensive signals, adaptive immunity utilizes far more specific antigen receptors. These antigen receptors are expressed by B and T cells, and function to better discriminate various antigenic epitopes in order to achieve specific immunity and immunological memory (1, 2).

Bone marrow

Hematopoiesis is the process of production of mature blood cells, which primarily takes place in the bone marrow (3). Here, pluripotent stem cells reside that give rise to the various types of blood cells. Pluripotent stem cells maintain, in contrast to differentiated blood cells, their proliferative ability throughout the life of an individual. These pluripotent stem cells divide to produce two types of stem cells. A common myeloid progenitor that gives rise to the myeloid lineage, which comprises erythrocytes, platelets and most of the cells of the innate immune system, such as granulocytes, macrophages, mast cells, monocytes, and dendritic cells. A common lymphoid progenitor gives rise to the lymphoid lineage, which comprises the natural killer cells of the innate immunity and lymphocytes of the adaptive immune system. There are two types of lymphocytes, B lymphocytes (B cells) and T lymphocytes (T cells) (4).

Normal B cell development

In the body, the humoral immune response is mediated primarily by B cells and requires enormous variability in immunoglobulins to deal with all possible antigens. Immunoglobulins, or antibody molecules, consist of a constant region and a variable region. The constant region takes one of only five distinguishable forms which determine the effector function. The variable region is the actual antigen binding site and can be composed of an apparently endless variety of different amino acid sequences, forming subtle differences that allow antibodies to bind specifically to an equally infinite variety of antigens. In addition, multiple regulatory mechanisms are required to discriminate self from non-self and to yield long-lasting immunological memory.

B cells arise in the bone marrow from stem cells and differentiate through a complicated and highly regulated process into cells that can produce antibodies capable of recognizing specific foreign antigens. The development of B cells in the bone marrow is initiated by the assembly of genes for the variable regions of the heavy and light chains of antibodies in B cell progenitors which is obligatory for B cell antigen receptor (BCR) expression (5). The BCR is a membrane bound immunoglobulin and consists of two heavy and two light chains. First, the heavy chains of the BCR are produced, thereafter the light chains (6).
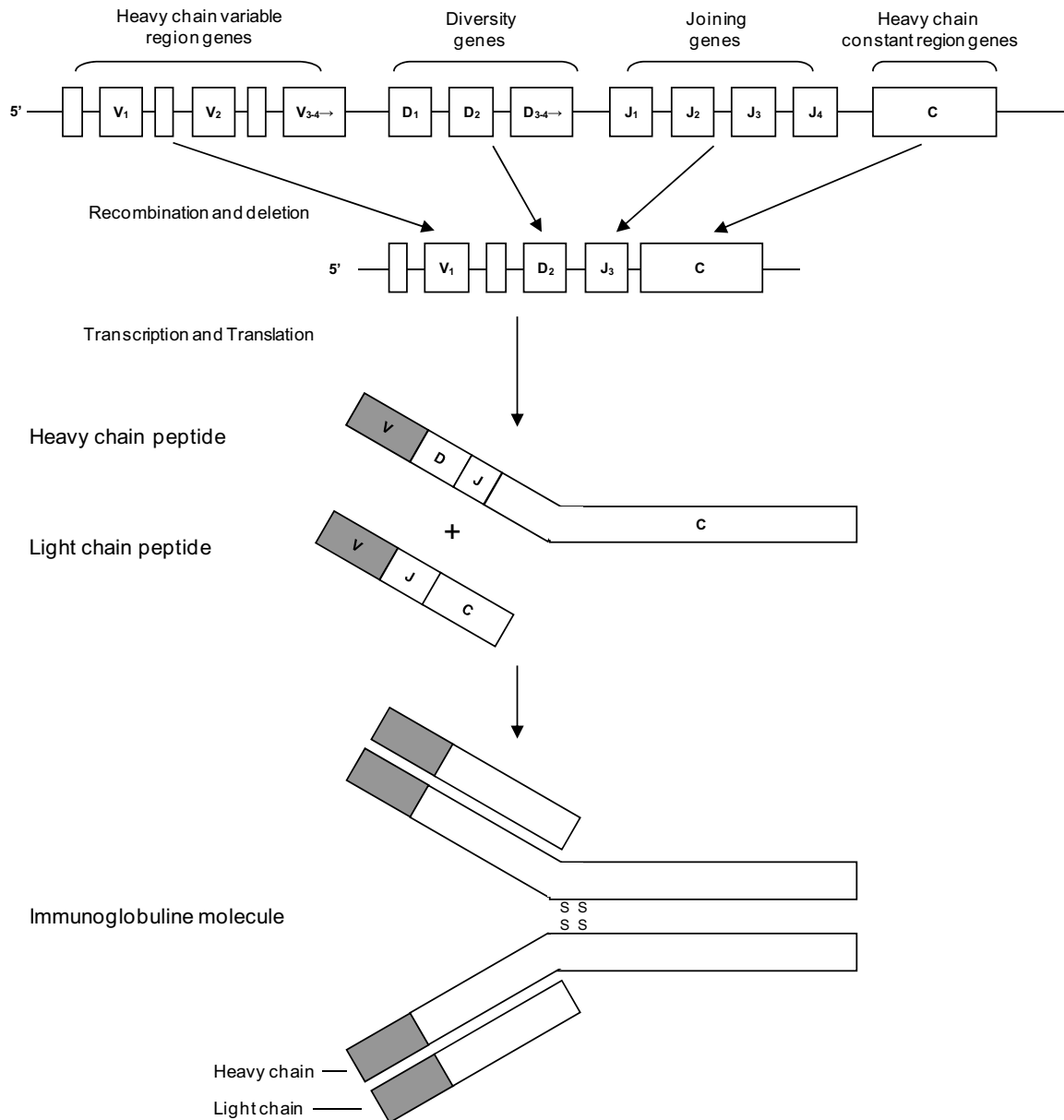
The variable regions of heavy and light chains are formed by DNA rearrangement of multiple different segments on the heavy and light chain loci (Fig. 1). This process is called V(D)J recombination, in which the gene rearrangements start at the heavy chain locus in pro-B cells with first random combinatorial joining of the D (diversity) and J (joining) gene segments (7). Subsequently, rearrangements of V (variable) gene segments to DJ-rearranged segments are induced and if successful at one allele, the other allele is turned off by allelic exclusion (8). Light chains consist of and rearrange V and J gene segments and are combined with the heavy chain. The recombination process generates an enormous antigen receptor diversity, which is further increased by random nucleotide introduction in the joining regions during recombination. An intact pre-BCR is expressed at the cell surface of the dividing pre-B cell.

B cell selection

During B cell proliferation, positive and negative selection checkpoints are set to test the competence of the (pre-)BCR (Fig. 2). In the bone marrow, positive selection occurs in cells expressing a transmembrane pre-BCR. This selection means that signaling from the pre-BCR is required to suppress V(D)J recombination and an appropriate pre-BCR signals for proliferative expansion (9). Whether pre-BCR cross-linking by an unknown ligand or simple surface expression is sufficient is still debated. Pre-B cells that fail to fulfill proper receptor requirements are developmentally arrested or forced into apoptosis (10). Thus, B cells expressing an appropriate pre-BCR will continue to develop. These immature B cells undergo negative selection at later stages of development, since most of the generated BCRs are self-reactive (11). This negative selection occurs before the immature B cells leave the bone marrow; if a BCR reacts with self-antigen in the bone marrow, receptor editing is induced. The process of receptor editing rescues the B cells when

secondary V(D)J rearrangements induce the replacement of the autoreactive antigen receptors to non-self reactive antigen receptors (12, 13). Immature B cells progress from a receptor-editing competent, apoptosis-resistant stage into a receptor-editing incompetent, apoptosis-sensitive stage (14). A B cell that passes

Figure 1. The heavy chain genes have no complete exon encoding the variable region domain, instead this is split into arrays of gene segments. Light chain genes are similarly organized on different chromosomes but they have no diversity gene segments. Immunoglobulin genes rearrange segments with the looping out of intervening DNA. This is done in a precise order: first the heavy chain rearranges, then if a functional heavy chain (always IgM initially) results (many joins are out of frame), the light chains rearrange also in order, first kappa then if kappa is unproductive (or cannot pair with the heavy chain) lambda.

both positive and negative selection checkpoints expresses a functional, non-self reactive BCR that is able to encounter foreign antigen as it leaves the bone marrow. The rearranged genes code for both the BCR at the cell surface and for secreted immunoglobulins after the B cell has differentiated into plasma cell. Thus, the BCR and the produced antibodies of one B cell have the same specificity for a particular antigen.

Germinal center reaction

After leaving the bone marrow, the immature B cells pass from the blood into secondary lymphoid organs: lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT). Immature B cells that migrate from the bone marrow to the periphery are referred to as transitional B cells. Transitional B cells can be distinguished from mature B cells by the absence of the ATP-binding cassette (ABC)B1 transporter (15) and because they express low levels of recombination activating gene (RAG) mRNA (16). Transitional B cells are short-lived and only 10−30% of these cells become long-lived mature, naïve B cells and are localized together with follicular dendritic cells (FDCs) in discrete clusters called primary follicles. These FDCs are thought to provide signals essential for the survival and continued recirculation of the naïve B cells. FDCs have taken up foreign antigen, which is presented on their membrane. B cells monitor the antigens present on FDCs and upon recognition of the antigen by their BCR the germinal center reaction is initiated (17). In addition to FDCs recently identified 'antigen transport cells' and mariginal zone B cells in the spleen have been implicated in the process of antigen presentation to B cells (18).

During the germinal center reaction the activated B cells start to divide forming a secondary follicle, the germinal center. Here their genomic DNA may undergo modifications, a process called somatic hypermutation (SHM). During SHM, small changes, mainly single nucleotide exchanges but also deletions and duplication, are introduced at a high rate into the variable-region genes of the BCR. Because of these changes a wide variety of B cells is made, all recognizing the same antigenic epitope but with a different affinity. B cells with a BCR with the highest affinity for the antigen are positively selected for survival (19). Depending on the antigen, the BCR can acquire another constant region in a process called class switch recombination (CSR) (20) by switching from IgM expression to heavy chain expression of other immunoglobulin classes: IgG, IgA or IgE, leaving the antigen

specificity unaltered (21). The subclass of the constant region confers functional specialization on the antibody: e.g. IgG1-3 and IgM can activate the classical pathway of complement activation, IgA1 the alternative pathway of complement activation, IgG1-3 can be transferred across the placenta, IgE has high-affinity binding to mast cells and basophils and, except for IgG2 and IgM, all (sub)classes can bind Fc receptors on macrophages and phagocytes. IgD is coexpressed with IgM on the surface of almost all mature B cells, although this isotype is secreted in only small amounts of plasma cells and its function is unknown. Although germinal center formation strongly facilitates CSR and SHM, other environments can also support CSR and SHM. B cells from patients of which the CD40 ligand gene is mutated (X-linked hyper-IgM syndrome type I), have a certain level of hypermutation, although there are no germinal centers (22). Also, T-cell-independent antigens initiate IgA class switching by linking B cells with multiple innate immune pathways. Whereas some T-cell-independent antigens activate B cells through Toll-like receptors (TLRs) (e.g. LPS), others activate B cells through their BCR (e.g. polysaccharides). T-cell-independent antigens can also provide additional B-cell-stimulating signals through DCs, which release soluble class-switch-inducing factors related to CD40L, including B-cell activating factor (BAFF; also known as BLyS) and a proliferation-inducing ligand (APRIL) (reviewed in (23)).

Terminal B cell differentiation

B cells that have successfully bound antigen and survived selection leave the germinal center and migrate into the periphery, where they become either memory B cells or plasma cells (24, 25). IL-10 has been put forward as the key cytokine that terminates the expansion of memory B cells by inducing differentiation into plasma cells (26). Plasma cells predominantly migrate to the bone marrow and have different live spans. Productive signaling events lead to the generation of short-lived antibody-secreting cells that survive only for a few weeks (27). Memory B cells reside in secondary lymphoid organs and rapidly proliferate and differentiate upon exposure to the same antigen without further involvement of germinal centers, generating high amounts of long-lived immunoglobulin secreting plasma cells that account for the persistence of the humoral immune response (28).
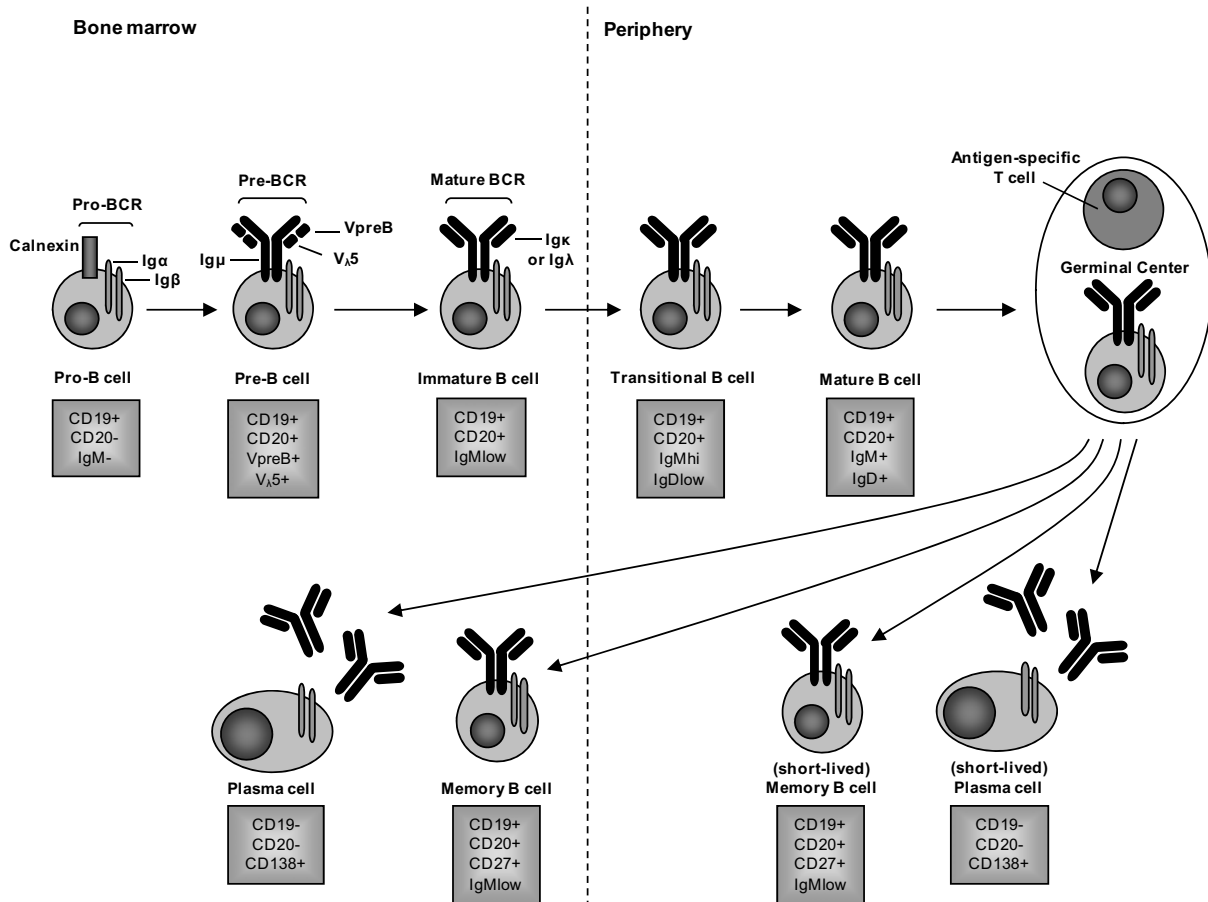
Figure 2. B-cell development occurs in both the bone marrow and peripheral lymphoid tissues such as the spleen. In the bone marrow, development progresses through the pro-B-cell, pre-B-cell and immature-B-cell stages. During this differentiation, rearrangements at the immunoglobulin locus ultimately result in the expression of a mature BCR that is capable of binding antigen. At this immature stage of development, B cells undergo a selection process to prevent any further development of self-reactive cells. Both receptor editing and clonal deletion have a role at this stage. Cells successfully completing this checkpoint leave the bone marrow as transitional B cell, eventually maturing into mature follicular B cell (or marginal-zone B cell). Following an immune response, antigen-specific B cells develop into either plasma cell or memory B cell.

Salmonella infection

Antibodies produced by antigen-specific B cells are important in the clearance of bacterial infections. On the one hand they opsonize bacteria to activate the complement system that will ultimately lead to lysis of the bacteria. On the other hand, antibodies opsonize bacteria to be recognized by phagocytic cells and subsequent destruction of the bacteria by phagocytes. *Salmonella enterica* is a Gram-negative, enteric pathogen responsible for disease syndromes of significant morbidity and mortality (29). After oral uptake, the bacterium crosses the intestinal

epithelium via M cells (or microfold cells). M cells are found in the follicle-associated epithelium of the Peyer's patch and differ from normal enterocytes in that they lack microvilli on their apical surface. Instead, M cells possess broader microfolds and the filamentous brush border glycocalyx, an extracellular polysaccharide layer found throughout the intestine attached to enterocytes, is much thinner or absent on M cells. This allows M cells to sample antigen/bacteria from the lumen and deliver it via transcytosis to the Peyer's patches (30). Another way of bacterial invasion in the intestinal mucosa is via dendritic cells (DCs). DCs express tight-junction proteins, open the tight-junctions between epithelial cells and send dendrites outside the epithelium into the lumen to directly sample microorganisms. In this way, DCs can transport bacteria to the basolateral side while preserving the integrity of the epithelial barrier (31). The bacteria are ultimately internalized by macrophages, dendritic cells, and neutrophils (32, 33). Entry into these cells is actively induced by the bacterium through an impressive array of effector proteins that orchestrate uptake by manipulating the host cellular machinery (34). *Salmonellae* manipulate host cells upon infection in order to alter the actin cytoskeleton allowing phagosomal cup formation and entry of the relatively large pathogen into the host cell. Bacterial effector proteins are therefore introduced into the host cytosol through the *Salmonella* Type III Secretion System (TTSS). After invasion, *Salmonella* forms an intracellular vacuole called the *Salmonella*-containing vacuole (SCV). Here another set of effectors is secreted into the host cytosol for vacuole maintenance and interference with the endosomal system to obtain nutrients and to prevent maturation and fusion with lysosomes (35, 36). *Salmonella* replicates in an expanding SCV (37, 38) and escapes detection by the immune system (39, 40). This feature of *Salmonella* is considered crucial for their survival and pathogenicity (41). Although *Salmonella* replicates in the phagosome, it remains unclear how they are released from the infected cell. This may follow from apoptosis or necrosis of the infected cell, but this is not established.

When *Salmonella* has passed the intestinal epithelium, it spreads via mesenteric lymph nodes to liver, bone marrow and spleen where replication continues (42). How *Salmonella* reaches these organs is unclear. So far, especially neutrophils, CD18-expressing phagocytes and DCs have been implicated (43, 44). Being facultative intracellular pathogens, immunity to *Salmonella* requires adequate humoral and cell-mediated immune responses (45, 46).

Antigen presentation by MHC class II molecules after BCR recognition

Antigen presentation by B lymphocytes is needed to generate high-affinity Abs (5, 47). Development of an effective humoral immune response is mediated by two actions of the BCR: transmembrane signaling through BCR-complexes to induce B cell differentiation and antigen internalization for processing followed by MHC class II-mediated presentation to acquire T cell help. The proper execution of both actions requires binding of a polyvalent antigen to multiple BCR molecules on one B cell.

Since primary B cells are not classical phagocytic cells, it is unclear how they acquire antigens from bacteria for antigen presentation. B cells can present particulate antigens in the context of MHC class II (48-51) and are able to extract antigen from a non-internalizable surface (52). Indeed, many B cell antigens are polyvalent as they are present in multiple copies to the particulate surfaces of microbes or cells (53). B cells use their BCR to concentrate specific antigen to the antigen loading compartments (termed MIIC for MHC class II containing compartment, see later) for loading of antigenic fragments onto newly synthesized MHC class II molecules (53). Besides internalization of antigen, the BCR drives intracellular targeting by accelerating the delivery of antigen to MIICs (54). Furthermore, BCR signaling ignited by antigen induces acidification of the MIICs which favors antigen loading onto newly synthesized MHC class II molecules (55). Together, these cellular adaptations enable B cells to preferentially present specific antigens that have been internalized via the BCR to CD4^{+} T cells. However, the exact regulation of MHC class II antigen presentation after BCR-specific recognition of particulate antigens is not clear.

The regulation of MHC class II antigen presentation

Regulation of the MHC class II antigen presentation pathway may affect the efficacy of MHC class II antigen presentation. MHC class II molecules are heterodimeric cell surface glycoproteins that present antigens to CD4+ T cells. After synthesis, the α and β subunits of the MHC class II molecule associate in the endoplasmic reticulum (ER) together with the invariant chain (Ii or CD74) (Fig. 3). The Ii folds in part through the antigen binding groove of the MHC class II molecule, stabilizing the αβ heterodimer and preventing the binding of ER polypeptides. Another function of Ii is to direct MHC class II molecules to the lysosomal-like MIIC compartments (56), where the majority of MHC class II antigen loading occurs, as it contains two short

leucine-based sequences in the cytoplasmic tail that are responsible for trafficking through the endocytic pathway (57). During transport to the MIIC compartments, the Ii is progressively degraded by various proteinases depending on the cell type (58), leaving only a small fragment (class II-associated invariant chain peptides (CLIP)) in the class II peptide binding groove (59). The dissociation of the CLIP peptide and subsequent loading of antigenic peptide is an essential step in antigen presentation. Release of CLIP is facilitated by the specialized chaperone HLA-DM (DM) (60), a nonclassical MHC molecule composed of an α and β subunit. In addition to displacing CLIP, DM catalyses the natural process of peptide dissociation (61) by associating to DR to generate a peptide-receptive conformation by opening the peptide-binding groove. DM dissociates the peptide-MHC class II complex that it recognizes by perturbing a critical hydrogen bond between a conserved histidine residue on the β-chain of the MHC class II molecule and the peptide backbone (62). DM thus releases both CLIP and other nonstable binding peptides. Consequently, DM acts as a peptide editor, favoring presentation of high affinity peptides (63-66). HLA-DO (DO), another nonclassical αβ heterodimer, is expressed in human B cells (also in thymic medullary epithelium (67) and in subsets of DCs (68)). Association with DM is necessary for efficient exit of DO from the ER and for accumulation in lysosomal vesicles (69). DO acts as a negative modulator of antigen loading by inhibiting the catalytic action of DM on class II peptide loading (70, 71). As a result, cells expressing DO show elevated cell surface levels of CLIP on HLA-DR3 molecules, paralleled by a reduced, but not abolished, presentation of antigenic peptides (70). DO does not act as a simple inhibitor of DM, but modulates in a pH-dependent manner. The activity of DM itself is optimal at pH 5.0, but it still catalyzes class II-peptide loading at pH 6.0. Association of DO with DM still allows a respectable amount of DM function at lysosomal-like pH, but abolishes it completely at pH 6.0, the pH of early endosomes. Thus, DO acts as a sort of pH sensor to control the activity of DM (72, 73). Thus, DO reduces MHC class II-mediated presentation of antigenic peptides in general and modulates the antigenic peptide repertoire by facilitating presentation of particular antigens, while suppressing others. DO therefore both limits and skews the class II-associated antigenic peptide repertoire in B cells (70, 72, 74).

The relative levels of DR, DM and DO determine the efficacy of MHC class II antigen presentation together with cellular processes that determine antigen processing and

intracellular trafficking of antigens, DR, DM and DO and eventually trafficking of MHC class II molecules loaded with antigenic peptides to the cell surface.
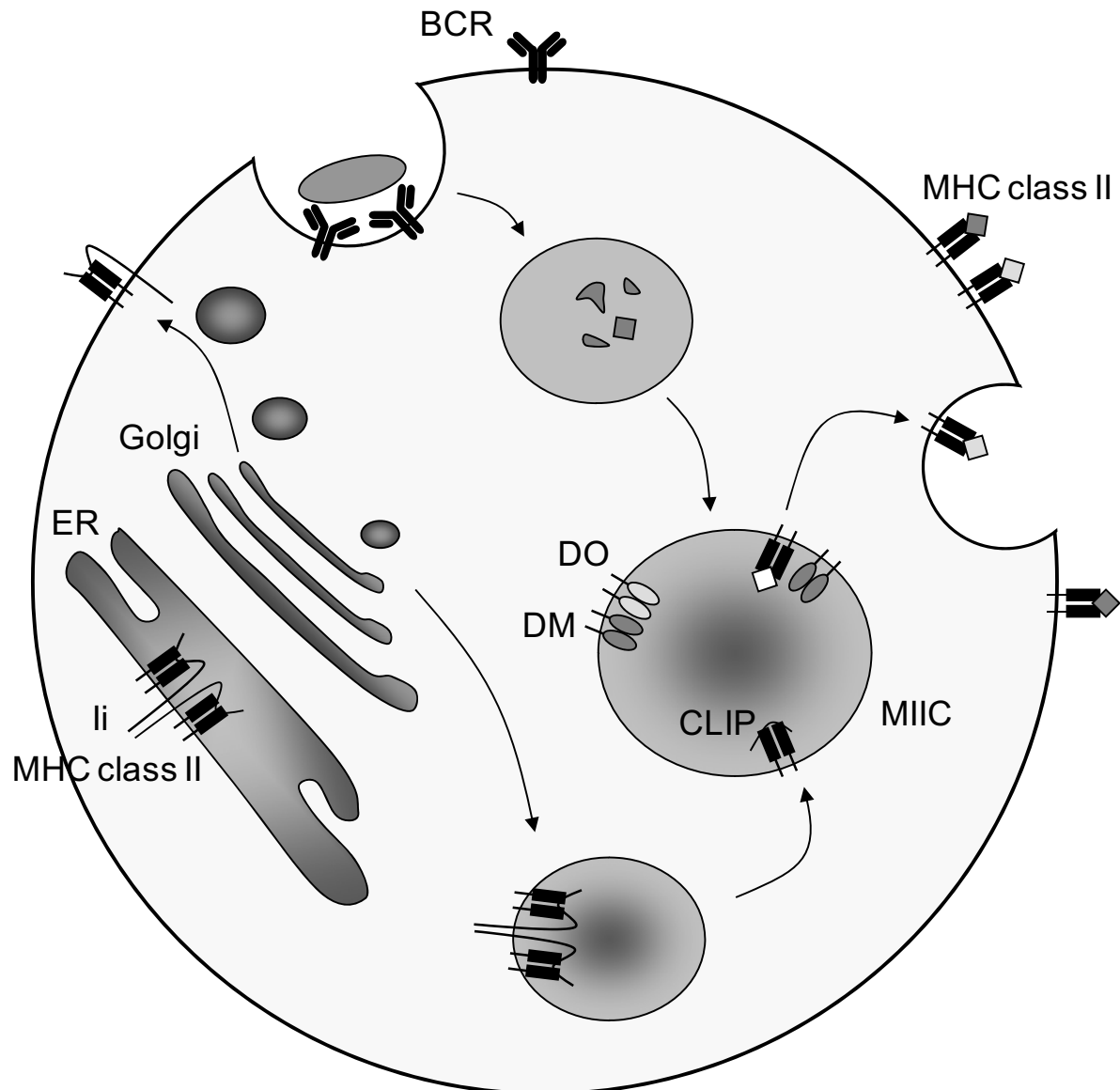
Figure 3. MHC class II αβheterodimers assemble in the endoplasmic reticulum (ER) with the assistance of invariant chain (Ii). The cytoplasmic tail of Ii contains a motif that targets the Ii–MHC class II complex to the endosomal/lysosomal pathway. During transport to the MIIC, Ii is degraded by a series of endosomal proteases with the CLIP fragment remaining, which prevents premature peptide loading. HLA-DM assists exchange of CLIP for relevant exogenous antigenic fragments in the MIICs prior to transport of stable MHC class II-antigen complexes to the plasma membrane. HLA-DO can inhibit DM-mediated peptide loading.

Regulation of MHC class II, DM and DO expression.

The relative expression of DO and DM (or the number of DM molecules in association with DO) related to the level or MHC class II expression control antigen presentation in B cells. Aberrant expression of DM, DO and/or DR could lead to an altered MHC class II peptide repertoire. Not surprisingly, DO and DM expression are very consistent and tightly regulated at different cellular levels in healthy B cells (75) and during B cell differentiation (76, 77). Expression of DR, DM and DO is regulated at the transcriptional as well as the post-translational level.

Transcription of MHC class II, DM and DO is regulated by a master regulator termed the class II transactivator (CIITA) (78, 79). CIITA is transcriptionally controlled by four distinct promoters, each transcribing a unique first exon and yielding a unique CIITA transcript (80). The promoters I, III and IV are differentially used in different cell types and in response to inflammatory stimuli. A physiological role for CIITA-PII is questioned as transcripts originating from this promoter are rare. CIITA-PI is constitutively active in myeloid dendritic cells (DCs) and CIITA-PIII constitutively in B cells, plasmacytoid DCs, monocytes and activated T cells (81). CIITA-PIV has been shown to be the promoter predominantly involved in IFN-γ-inducible CIITA expression (82, 83). In healthy B cells transcription of the MHC class II genes is tightly regulated by CIITA, but dysregulation has been observed in tumors (84, 85).

MHC-mediated antigen presentation and cancer

A successful anti-tumor T cell response is achieved by the two effector arms of the immune system. On the one hand, CD8^{+} CTLs ensure specific elimination of tumor cells upon recognition of MHC class I-antigen complexes. On the other hand CD4^{+} T helper cells generate the required T cell help upon activation by MHC class II-antigen complexes (86). The absence of T cell help can lead to abortive induction of CTL responses and subsequent tolerance (87). Moreover, T cell help is required for the maintenance of CTL responses, which is essential in diseases like cancer (88, 89). It has been shown that CD4^{+} T cell inclusion in adoptive T cell transfer studies improved tumor clearance by the CD8^{+} T cells (90, 91). MHC class II molecules play a pivotal role in the induction and regulation of an antigen-specific immune response. MHC class II antigen presentation activates antigen-specific CD4^{+} T cells. Over the last years it has become clear that the establishment of an effective CD4^{+} T cell response is required for both the induction and maintenance of anti-tumor CD8^{+} cytotoxic T lymphocytes (CTL) responses (92). Indeed, loss of MHC class II

expression has been observed in diffuse large B cell lymphomas with fewer tumor-infiltrating CD8+ T cells in MHC class II-negative tumors (93, 94).

B-cell chronic lymphocytic leukemia

During B cell differentiation, uncontrolled growth of B cells can occur, resulting in the formation of lymphomas or leukemia.

B-cell chronic lymphocytic leukemia (B-CLL) is the most common form of leukemia in adults in the Western world. Typical for the disease is the highly variable clinical course, with survival rates varying between a few months and two decades (95). B-CLL is characterized by a progressive accumulation of a malignant B cell population that fails to undergo apoptosis. Apparently the immune system is unable to deal with this abnormal cell population. Indeed, B-CLL is characterized by striking immune incompetence in which not only the number but also the function of the B and T cells is impaired (96). Prognostic factors for survival of B-CLL patients are the mutational status of the immunoglobulin *IGHV* genes, cytogenetic aberrancies, CD38 and ZAP-70 expression (97). Today, the strongest predictor for survival in B-CLL is the mutational status of the *IGVH* genes.

MHC-mediated antigen presentation and B-CLL

In B-CLL patients, malignant B cells accumulate in the bone marrow and periphery. Apparently, these cells are not recognized by the immune system in such a way that the malignant cells are effectively cleared. This may be due to immune escape by the malignant cells by preventing CD4$^+$ T cell activation, since B-CLL cells lack significant expression of CD80 and CD86 costimulatory molecules (98). However, the absolute numbers of T cells are increased in B-CLL patients. Whether this expansion is a result of (altered) MHC class II antigen presentation by malignant B cells has not been established.

A shift in MHC class II antigen presentation by B-CLL cells may lead to altered T helper cell activation and subsequent help to CD8$^+$ CTLs. It is unclear whether the T cell expansion in B-CLL is indicative for attempted but unsuccessful tumor clearance or contributes in another way to the disease, for instance by creating an environment that supports survival of neoplastic cells (99). Antigen-independent mechanisms have been implicated in the T cell expansion in B-CLL (reviewed in (100)). The TCR-dependent oligoclonal/monoclonal expansion of CD4+ T cells in B-CLL however, points to an antigen-driven process. How malignant B cells present

antigens via MHC class II molecules to CD4^{+} T cells and whether this may be an explanation for observed T cell expansion in B-CLL is unclear. If so, modulation of these MHC class II responses would be an interesting approach to control these tumors.

Acute myeloid leukemia

Acute myeloid leukemia (AML) is a myeloproliferative disorder, characterized by an arrest in differentiation of hematopoietic stem cells due to acquired mutations. This results in accumulation of immature non-lymphoid cells in the bone marrow and peripheral blood, often accompanied by suppression of the normal blood cells. AML is a heterogeneous disease in which subgroups can be defined with different stages of immature blasts present and each group is characterized genetic lesions, clinical behavior, prognosis and therapy (101). With chemotherapy and stem cell transplantation approximately 70 % of patients achieve complete remission, but half of these patients relapse (102). Although AML blasts generally express MHC class II molecules (103), they have escaped the initial immune response in acute disease status. How efficient AML blasts present antigens via MHC class II molecules is unclear.

Scope of this thesis

In this thesis, the role of MHC class II antigen presentation in the immune response against bacterial infections and tumors was investigated.

MHC class II antigen presentation is indispensable for activation of CD4$^+$ T helper cells, which give help to B cells for antibody production. So far it is unclear how particulate antigens induce MHC class II antigen presentation and CD4+ T helper activation, as B cells are considered to be non-phagocytic. In **Chapter 2** we investigate how B cells deal with particulate antigens, using beads coated with antibodies specific for the BCR or the bacterium *Salmonella typhimurium* as model systems. We demonstrate that B cells phagoyctose the beads and *Salmonella* upon antigen-recognition by the BCR and that these B cells subsequently activate CD4$^+$ T cells. Since *Salmonella* is a facultative intracellular bacterium, we investigated the fate of *Salmonella* inside the B cell in **Chapter 3**. For elimination of infected cells, an effective induction of antigen-specific CD8+ T cells, which can kill infected target cells, is desirable. In **Chapter 4** we demonstrate that B cells are able to cross-present bacterial antigens via MHC class I to induce an effective CD8$^+$ T cell response. We also show that the ability of B cells to activate *Salmonella*-specific CD8$^+$ T cells requires help of CD4$^+$ T cells.

Induction of CD4$^+$ T cells is indispensable for the generation of an effective and long-lasting immune response. Some types of cancer escape immune surveillance by interfering with antigen presentation. Most AML blasts express MHC class II molecules on their cell surface which could induce CD4$^+$ T cells activation. In **Chapter 5** we analyzed cell surface expression of HLA-DR and the efficacy of MHC class II antigen presentation by analyzing the amount of the self-peptide CLIP inside the peptide binding groove of HLA-DR. **Chapter 6** describes the efficacy of MHC class II antigen presentation in B-CLL and we correlated this to the *ex vivo* analysis of the T cell compartment in B-CLL patients. Evaluation of the MHC class II antigen presentation components at the mRNA level in **Chapter 7** revealed that all components are significantly higher transcribed in B-CLL patients. Survival analysis of patients showed that the level of *DOA* mRNA at the time of sample acquisition has prognostic power. **Chapter 8** contains a general discussion and a summary of the results.

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